



TITLE:

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Fgf20b is required for the ectomesenchymal fate establishment of cranial neural crest cells in zebrafish

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ABSTRACT

In cranial skeletal development, the establishment of the ectomesenchymal lineage within the cranial neural crest is of great significance. Fgfs are polypeptide growth factors with diverse functions in development and metabolism. *Fgf20b* knockdown zebrafish embryos showed dysplastic neurocranial and pharyngeal cartilages. Ectomesenchymal cells from cranial neural crest cells were significantly decreased in *Fgf20b* knockdown embryos, but cranial neural crest cells with a non-ectomesenchymal fate were increased. However, the proliferation and apoptosis of cranial neural crest cells were essentially unchanged. *Fgfr1* knockdown embryos also showed dysplastic neurocranial and pharyngeal cartilages. The present findings indicate that Fgf20b is required for ectomesenchymal fate establishment via the activation of Fgfr1 in zebrafish.

Key words: Fgf; Fgf20b; cranial neural crest; pharyngeal arch; ectomesenchyme; zebrafish

1. Introduction

In vertebrate embryonic development, the formation of a head skeleton comprising the cranium and mandible is essential for the establishment of a proper physical frame. Most of the bones and cartilage in the head skeleton are derived from cranial neural crest cells [1]. After developing at the neural plate and epidermis, neural crest cells emigrate from the dorsal neural tube to their final destination during embryonic development. Cranial neural crest cells differentiate into ectomesenchyme and non-ectomesenchyme. In cranial skeletal development, the ectomesenchyme terminally differentiates into bones, cartilages, and associated connective tissues and generates much of the craniofacial skeleton. The establishment of the ectomesenchymal lineage within the cranial neural crest is of great significance [2].

Fgfs are polypeptide growth factors with diverse functions in development and metabolism. The Fgf family comprises 22 and 28 members in humans/mice and zebrafish, respectively [3, 4]. We originally identified Fgf20 as a neurotrophic factor that acts via the activation of Fgf receptor 1 (Fgfr1) in a paracrine manner [5, 6]. *Fgf20* is expressed in mouse embryonic calvaria and developing limbs [7]. In addition, Fgf20 is potentially required for sensory cell specification in mouse cochlear development [8]. Fgf20 also regulates the development of embryonic and neural stem cells into tyrosine hydroxylase-positive neurons in cultured cells [9, 10]. However, the physiological roles of Fgf20 remain unclear.

Zebrafish *Fgf20* is an ortholog of human/mouse *Fgf20*. Zebrafish *Fgf20a* and *Fgf20b* are paralogs [3, 11]. Fgf20a is required for fin regeneration, the restriction of neurogenesis, and the segment center progenitor population [11, 12]. However, the roles of Fgf20b remain unclear. In this paper, we report that Fgf20b is required for the ectomesenchymal fate establishment of cranial neural crest cells in zebrafish.

2. Materials and methods

2.1. Fish maintenance

Zebrafish (*Danio rerio*) were maintained and the developmental stages of embryos were determined as described [13].

2.2. Whole mount in situ hybridization and staining

Whole mount in situ hybridization was performed as described [13]. The *Fgf20b* probe was synthesized using a full-length cDNA-containing plasmid. Other probes used were zebrafish *foxd3* [14], *sox10* [15], *dlx2* [16], *col2a1a* [17], and *Fgfr1* [13]. Cartilage was stained with Alcian blue as described [18].

2.3. Morpholino injection

Antisense MOs were synthesized by Gene-Tools, LLC (Corvallis, OR). The sequences were as follows: splice-site-targeted *Fgf20b* MO1, 5'-AAGACAAGTCTGCTTACTGACCAT-3'; splice-site-targeted *Fgf20b* MO2, 5'-TTCCAAAATACCTGGAGAAGAATAA-3'. A splice-site-targeted *Fgfr1* MO was used as described [13]. The *Fgf20b* MO1 (20 or 30 ng), *Fgf20b* MO2 (4 or 6 ng), and *Fgfr1* MO (12 ng) were injected into one- to two-cell embryos. To examine each MO's efficacy, RNA was isolated from wild-type, *Fgf20b* MO1-injected or *Fgf20b* MO2-injected embryos. cDNA was amplified from the RNA by RT-PCR.

2.4. Rescue experiments

Capped zebrafish *Fgf20b* sense mRNA was prepared with the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's directions from linearized *pCS2+* plasmids containing zebrafish *Fgf20b* cDNA. To perform rescue experiments, 0.05 pg of *Fgf20b* sense mRNA was injected separately, immediately after the injection of *Fgf20b* MO1 (20 ng) into two-cell embryos.

2.5. Detection of proliferating and apoptotic cells

Proliferating cells were detected by immunohistochemistry using an anti-phosphorylated H3 (H3P) antibody (Upstate Biotechnology). Apoptotic cells were examined by TUNEL assay using the DeadEnd colorimetric detection kit (Promega) as described.

3. Results and discussion

3.1. Spatiotemporal expression pattern of *Fgf20b*

We examined the spatiotemporal expression pattern of *Fgf20b* in zebrafish embryos by RT-PCR (Fig. 1A) and whole mount in situ hybridization (Fig. 1B-G). *Fgf20b* expression was barely detectable at 12 hpf. However, the expression increased gradually until at least 120 hpf. This expression pattern is similar to that of *Fgf20a*. At 14 hpf, *Fgf20b* was expressed in the region where migrating cranial neural crest cell subpopulations possibly exist. *Fgf20b* was expressed in the pharyngeal arch at 20 hpf and further in the mandibular arch (the first pharyngeal arch), hyoid arch (the second pharyngeal arch), gill arches, and the otic vesicle at 36

hpf. The pattern for *Fgf20b* is quite distinct from that for *Fgf20a*, which is mainly expressed in the hindbrain at 16 hpf and a cluster of cells in each rhombomere after 18 hpf [12]. Cranial neural crest cells arise from dorsal and lateral regions of the neural ectoderm, and then migrate to the pharyngeal arches, where they form neural and skeletal tissues [18]. The expression of *Fgf20b* suggests potential roles in the development of cranial neural crest cells.

3.2. Inhibition of *Fgf20b* functions results in mandibular hypoplasia

To inhibit *Fgf20b* functions in zebrafish embryos, we used splice-site-targeted *Fgf20b* MOs (*Fgf20b* MO1 and MO2) (Fig. S1A). RNA was isolated from wild-type, *Fgf20b* MO1-injected, or *Fgf20b* MO2-injected embryos at 48 hpf. *Fgf20b* cDNA was amplified from the RNA by RT-PCR using the P1 and P2 primers (Fig. S1B). Mature *Fgf20b* mRNA levels decreased significantly in the *Fgf20b* MO1 and *Fgf20b* MO2-injected embryos in a dose-dependent manner. In addition, mis-spliced *Fgf20b* mRNA forms were significantly detected. These results indicate that *Fgf20b* MO1 and MO2 significantly impaired the maturation of *Fgf20b* mRNA. We analyzed *Fgf20b* knockdown embryos at 5 days post fertilization (dpf) (Fig. 2A-H). The *Fgf20b* MO1-injected embryos showed impaired head regions (75.6%, n=45 by 20 ng; 83.3%, n=24 by 30 ng). In particular, mandibular hypoplasia, a significant reduction in the size of the lower jaw, was observed. The *Fgf20b* MO2-injected embryos showed similar phenotypes to the *Fgf20b* MO1-injected embryos (86.1%, n=36 by 4ng; 98.7%, n=44 by 6 ng). However, the mandibular hypoplasia in *Fgf20b* MO1(20 ng)-injected embryos was significantly rescued by the injection of capped *Fgf20b* mRNA (69.2%, n=26 by 0.025 pg mRNA; 83.3 %, n=24 by 0.05 pg mRNA). These results suggest that *Fgf20b* is required for the proper formation of the lower jaw.

3.3. *Fgf20b* is required for neurocranial and pharyngeal cartilage to form

We examined *Fgf20b* knockdown embryos at 5 dpf by histological analysis with Alcian blue, which stained sulfated glycosaminoglycan-rich tissues such as cartilage (Fig. 2I-N). The size of the anterior neurocranial cartilage, which includes the ethmoid plate and trabeculae, and pharyngeal cartilage was significantly reduced in *Fgf20b* knockdown embryos (75.6%, n=45 by 20 ng MO1; MO2: 86.1%, n=36 by 4 ng MO2). In particular, the first and second pharyngeal cartilages such as Meckel's cartilage, palatoquadrates, basihyal and ceratohyals were dysplastic. These results indicate that *Fgf20b* is required for the proper formation of neurocranial and pharyngeal cartilages.

3.4. *Fgf20b* is required for the ectomesenchymal fate establishment of cranial neural crest cells

Cranial neural crest cells have the potential to generate mesenchymal derivatives, which can be classified into two mesenchymes, ectomesenchyme and non-ectomesenchyme [19]. In zebrafish, these cells are spatially segregated in the premigratory neural crest cells [15]. After migrating to pharyngeal arches, ectomesenchymal cells form cartilage and connective tissues [2]. The drosophila distal-less homeobox gene *dlx2* is a marker for ectomesenchymal cells [2]. *dlx2* expression was significantly decreased in *Fgf20b* knockdown embryos (20 ng MO1) (78.8%, n=33) (Fig. 3A, B). The forkhead box-containing transcription factor gene *foxd3* is expressed in premigratory and early migratory neural crest cells [20]. The HMG box-containing transcription factor gene *sox10* is expressed in the migratory neural crest cells [2]. *foxd3* and *sox10* are markers for neural crest cells with a

non-ectomesenchymal fate [2]. *foxd3* and *sox10* expression was significantly increased in *Fgf20b* knockdown embryos (20 ng MO1) at 24 hpf (67.7%, n=31 and 77.4%, n=32, respectively) (Fig. 3C-F). These results indicate that the neural crest cells did not properly differentiate into ectomesenchymal cells in *Fgf20b* knockdown embryos

3.5. *Fgf20b* is not required for the endoderm to form in neurocranial and pharyngeal cartilages

We examined the roles of *Fgf20b* after the migration of cranial neural crest cells to the pharyngeal arches. The type II collagen gene *col2a1a* is a marker for differentiating chondrocytes [17]. *col2a1a* expression was also decreased in the pharyngeal arches of *Fgf20b* knockdown embryos (90.6 %, n=32) (Fig. 3G, H). These results indicate that a decrease in ectomesenchymal cells resulted in diminished chondrogenesis.

The pharyngeal endoderm interacts with the neural crest during the formation of neurocranial and pharyngeal cartilages [21]. The NK2 transcription factor gene *nkx2.3* is a marker for the pharyngeal endoderm [22]. *nkx2.3* expression in the pharyngeal endoderm was essentially unchanged in *Fgf20b* knockdown embryos (20 ng MO1) at 36 hpf (87.5 %, n=32) (Fig. 3I, J). The bHLH transcription factor gene *hand2*, which is expressed in the ventral endoderm during the pharyngeal arch's development, is required for the formation of the ventral pharyngeal cartilage including the lower jaw [23]. *hand2* expression was also essentially unchanged in the *Fgf20b* knockdown embryos (20 ng MO1) at 24 hpf (77.4 %, n=31) (Fig. 3K, L). These results suggest that *Fgf20b* is not essential to the formation of the endoderm in the pharyngeal cartilage.

3.6. *Fgf20b* is not critically involved in the proliferation and survival of cranial neural crest cells

In *Fgf20b* knockdown embryos, the number of cranial neural crest cells with a non-ectomesenchymal fate was increased, whereas the number of ectomesenchymal cells was decreased. Therefore, we examined the proliferation and survival of cranial neural crest cells in *Fgf20b* knockdown embryos. Proliferating cell levels examined by immunohistochemistry with phosphohistone-H3 antibody were essentially unchanged in *Fgf20b* knockdown embryos (20 ng MO1) at 14 and 24 hpf (Fig. 4A-H, Q), indicating that the proliferation of cranial neural crest cells was essentially unchanged in *Fgf20b* knockdown embryos. Apoptotic cell levels examined by the TUNEL assay were only slightly increased in *Fgf20b* knockdown embryos (Fig. 4I-P, R), indicating that the survival of cranial neural crest cells was not critically affected in *Fgf20b* knockdown embryos.

3.7. *Fgfr1* is required for neurocranial and pharyngeal cartilage to form

Fgf20 functions via the activation of *Fgfr1* in a paracrine manner [6]. *Fgf20b* is also expected to function via the activation of *Fgfrs* in a paracrine manner. The zebrafish *Fgfr* gene family comprises four members, *Fgfr1-Fgfr4* [13]. We generated *Fgfr1-Fgfr4* knockdown embryos injected with splice-site targeted *Fgfr1-Fgfr4* MOs as described previously [13]. The knockdown embryos at 5 dpf were examined by histological analysis with Alcian blue staining. Aberrant neurocranial and pharyngeal cartilages were observed only in *Fgfr1* knockdown embryos (70 %, n=10 by 12 ng) (Fig. S2A-D). *Fgfr1* was expressed in the region where migrating cranial neural crest cell subpopulations possibly exist at 14 hpf (Fig. S2E). Later, *Fgfr1* was expressed in the pharyngeal arches at 24 hpf (Fig. S2F).

This expression pattern is essentially consistent with that reported [24]. In addition, Fgf20 potentially binds to and activates Fgfr1 [6]. These results indicate that Fgf20b possibly functions via the activation of Fgfr1

Zebrafish Fgf20a functions in fin regeneration, the restriction of neurogenesis, and the segment center progenitor population [11, 12]. The present results indicate that Fgf20b has roles distinct from those of Fgf20a. In conclusion, Fgf20b contributes to head skeletal development by establishing the ectomesenchymal fate of cranial neural crest cells in zebrafish.

Acknowledgements

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Figure legends

Fig. 1. Spatiotemporal expression pattern of *Fgf20a* and *Fgf20b*. (A) The expression of *Fgf20a*, *Fgf20b*, and *eflα* as a control in embryos was examined by RT-PCR. (B-G) The expression of *Fgf20b* in embryos was examined by whole mount in situ hybridization. Arrows at 14 and 20 hpf indicate possible regions of migrating cranial neural crest cell subpopulations and the pharyngeal arch, respectively. pa1, the first pharyngeal arch (the mandibular arch); pa2, the second pharyngeal arch (the hyoid arch); ga, gill arches; ov, the otic vesicle. Lateral views (B, D, F), dorsal views (C, E, G).

Fig. 2. The effect of *Fgf20b* knockdown on morphogenesis. (A-F) Two-cell embryos injected with *Fgf20b* MO1 and *Fgf20b* MO2 at 5 dpf. In contrast to wild-type embryos, there was a significant reduction in the size of the lower jaw in *Fgf20b* MO1- or MO2-injected embryos. Arrows indicate the aberrant lower jaw. (G, H) The reduction in the size of the lower jaw in *Fgf20b* MO1 embryos was clearly prevented by the injection of *Fgf20b* mRNA. Lateral views of the embryos are shown. The head region in A, C, E and G is shown at a higher magnification in B, D, F and H, respectively. (I-N) The effect of *Fgf20b* knockdown on neurocranial and pharyngeal cartilages. Alcian blue stained cartilage elements at 5 dpf in wild-type embryos, *Fgf20b* MO1-injected, and *Fgf20b* MO2-injected embryos. In *Fgf20b* knockdown embryos, the size of ethmoid plate, trabeculae and pharyngeal cartilage were significantly reduced in size. Arrows indicate the anterior neurocranial cartilage including ethmoid plate, trabeculae and pharyngeal cartilage. bh, basihyal; ch, ceratohyal; e, ethmoid plate; m, Meckel's cartilage; pq, palatoquadrate; t, trabeculae. Lateral views (I, K, M); ventral views (J, L, N).

Fig. 3. The effect of *Fgf20b* knockdown on the commitment of cranial neural crest cells (A-F). (A, B) The expression of *dlx2* in embryos at 24 hpf. Arrows indicate the expression of *dlx2* in ectomesenchymal cells. The expression of *dlx2* was significantly decreased in *Fgf20b* knockdown embryos. (C-F) The expression of *foxd3* and *sox10* in embryos at 24 hpf. Arrows indicate the expression of *foxd3* and *sox10* in cranial neural crest cells with a non-ectomesenchymal fate. The expression of both *foxd3* and *sox10* was significantly increased in *Fgf20b* knockdown embryos. *sox10* was also expressed in the otic vesicle (ov). Dorsal views of embryos are shown. Chondrogenesis and endoderm formation neurocranial and pharyngeal arches in *Fgf20b* knockdown embryos (G-L). (G, H) The expression of *col2a1a*, which is required for chondrogenesis, in embryos.

Arrows indicate the expression of *col2a1a* in the pharyngeal arches. The expression of *col2a1a* was significantly decreased in *Fgf20b* knockdown embryos. (I-L) The expression of *nkx2.3* and *hand2*, markers for the pharyngeal endoderm, in embryos. The expression of *nkx2.3* and *hand2* was essentially unchanged in *Fgf20b* knockdown embryos. Lateral views (G, H, K, L); dorsal views (I, J).

Fig. 4. Proliferation and survival of cranial neural crest cells in *Fgf20b* knockdown embryos. (A-H) Proliferating cells, phospho-histone H3-positive cells, were examined. The regions between arrowheads indicate areas of migratory cranial neural crest cells (A, B) and pharyngeal arches (C, D). Arrows indicate phospho-histone H3-positive cells in the sections (E-H). (Q) The H3P-positive cells in the regions were examined quantitatively. (I-P) Apoptotic cell levels were examined by TUNEL assay. The regions between arrowheads indicate areas of migratory cranial neural crest cells (I, J) and pharyngeal arches (K, L). Arrows indicate apoptotic cells. (R) The apoptotic cells in the regions were examined quantitatively. Lateral views (A-D, I-L); sections of embryos (E-H, M-P). Error bars correspond to the standard deviation of the mean. Asterisk; $P < 0.01$ by Student's t test. The results are the mean \pm S.D. for 3 independent sections from 5 embryos

Fig.1

A

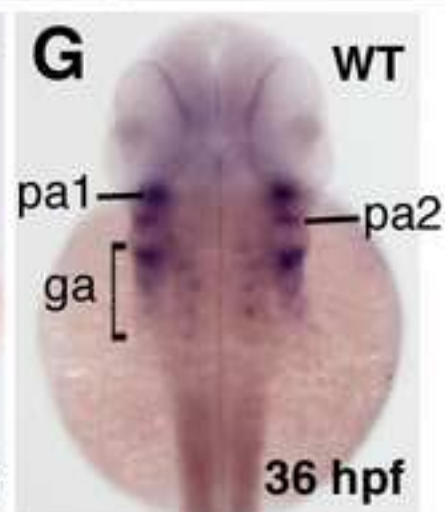
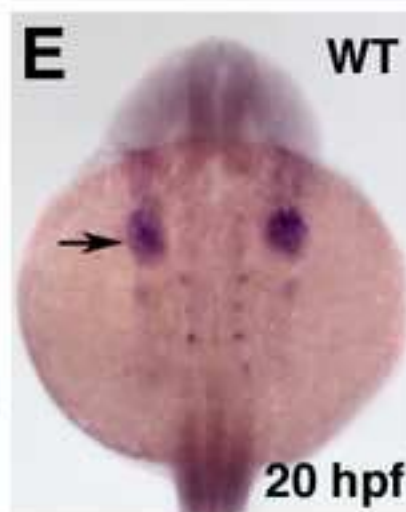
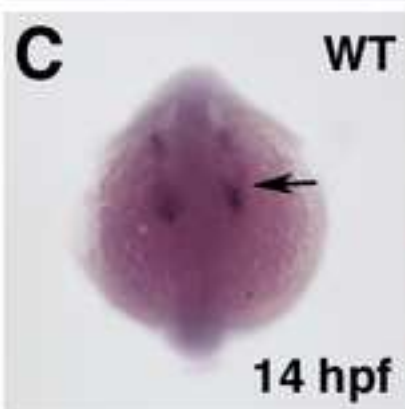
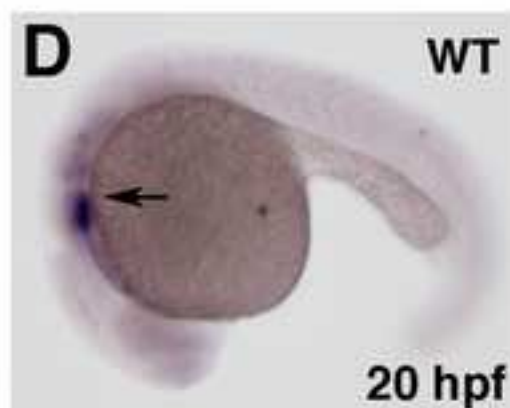
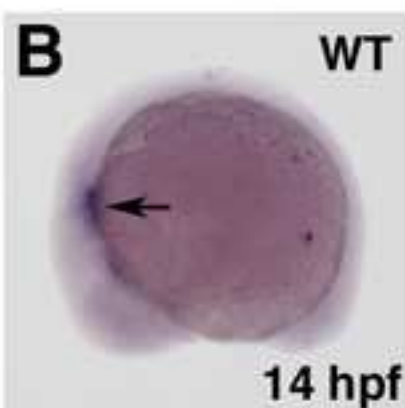
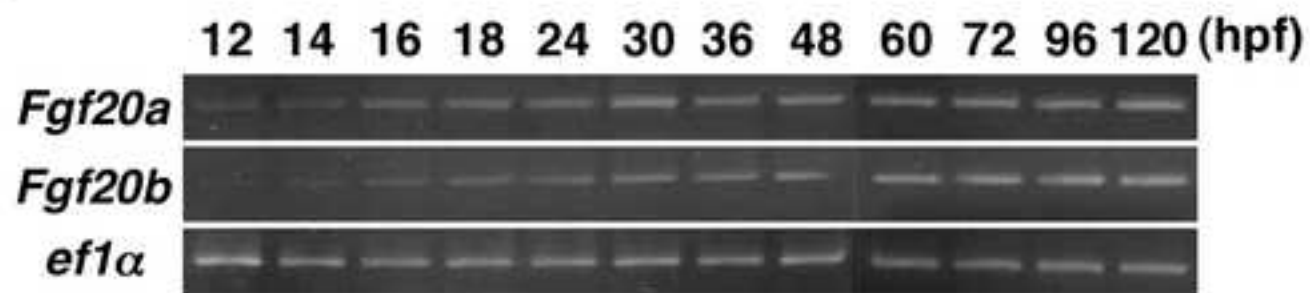


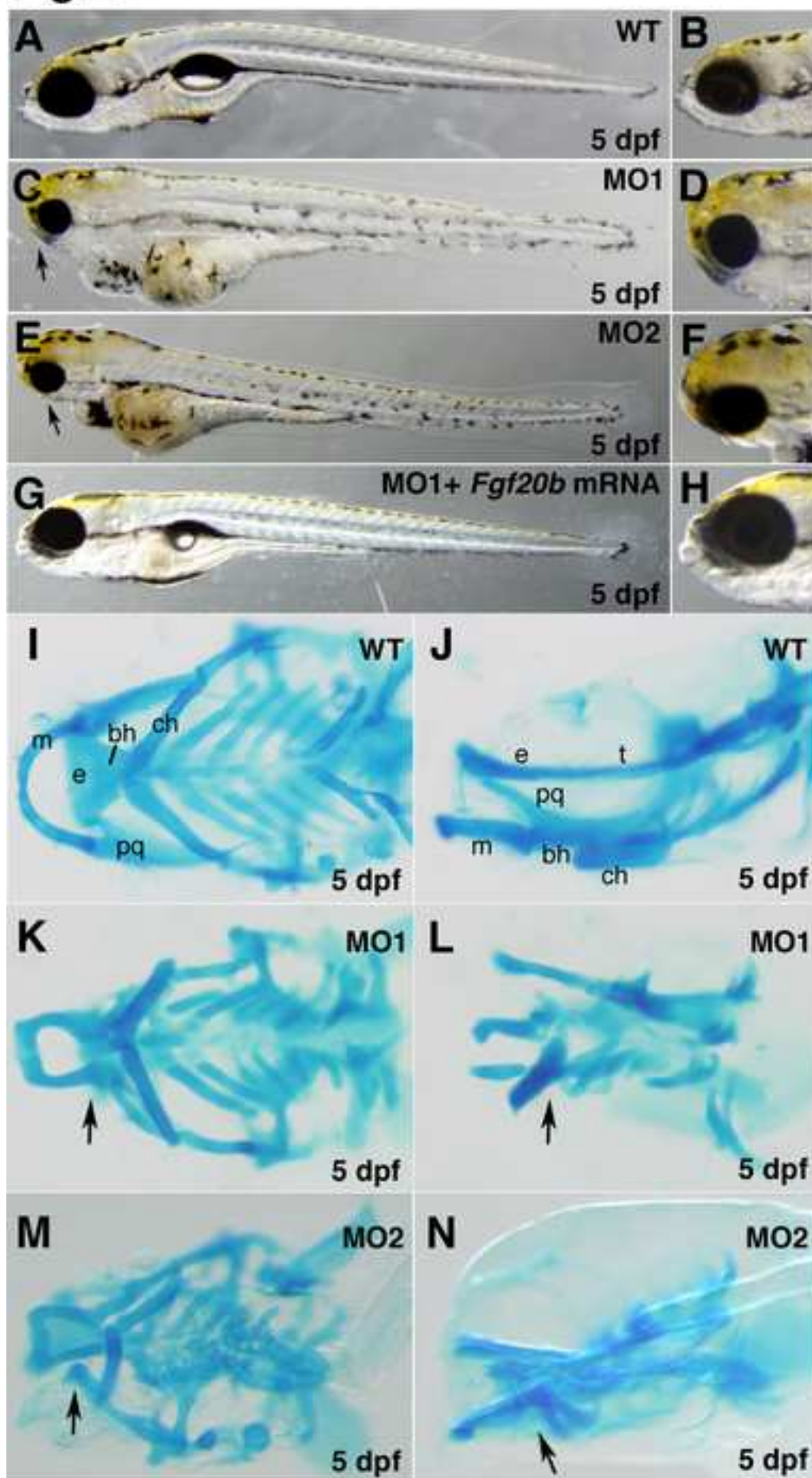
Fig.2

Figure 3
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Fig.3

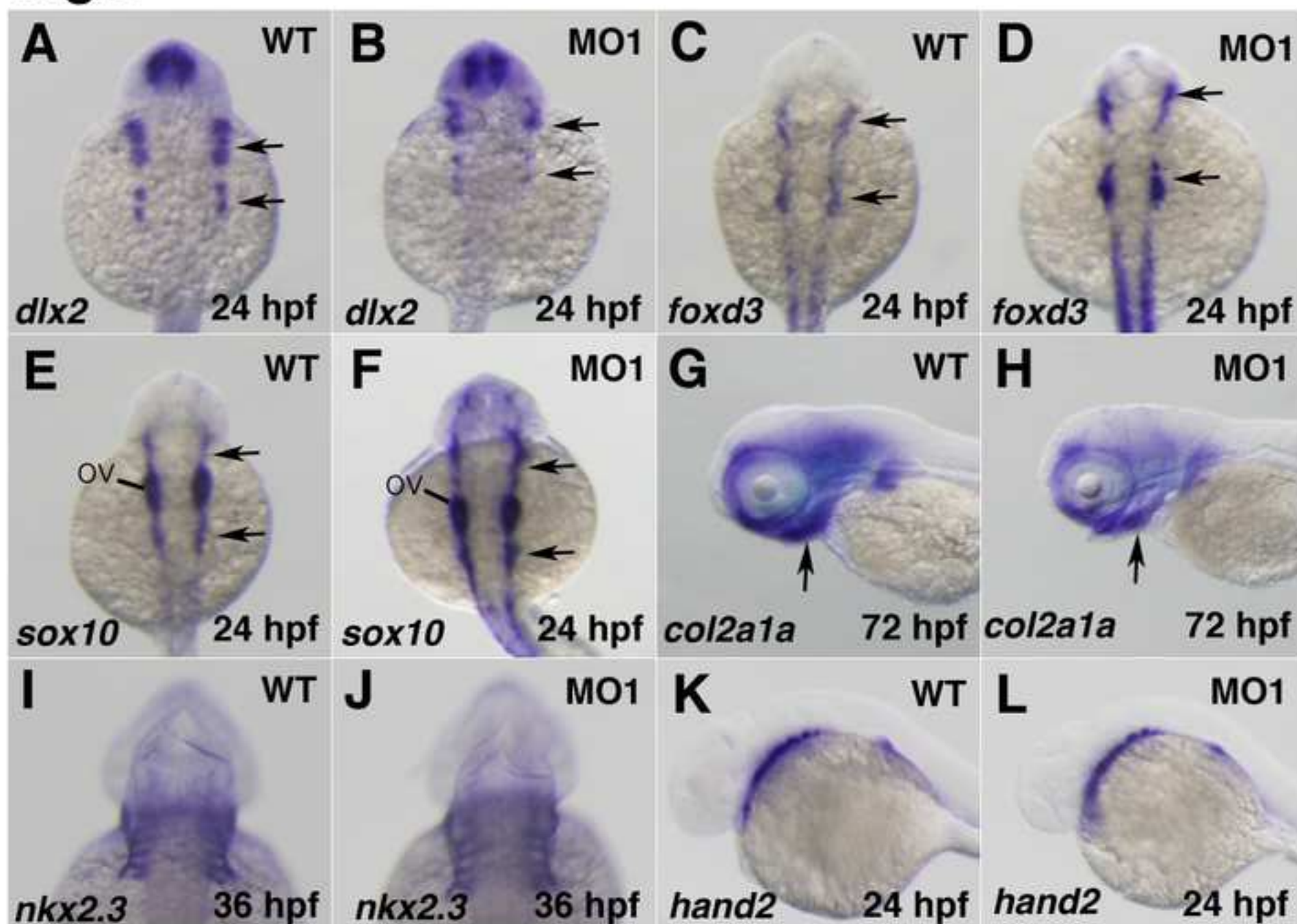


Fig.4

